

**Signal transduction mechanism for potentiation by  $\alpha_1$ - and  $\beta_2$ -adrenergic agonists of L-ascorbic acid-induced DNA synthesis and proliferation in primary cultures of adult rat hepatocytes**

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**Abstract:** We investigated the effects of  $\alpha$ - and  $\beta$ -adrenergic agonists on L-ascorbic acid-induced hepatocyte DNA synthesis and proliferation in primary cultures of adult rat hepatocytes. In addition, possible signal transduction mechanisms for the effects of  $\alpha$ - and  $\beta$ -adrenergic agonists of L-ascorbic acid-induced hepatocyte mitogenesis were examined. The results showed that phenylephrine ( $10^{-6}$  M) and metaproterenol ( $10^{-6}$  M) alone did not induce hepatocyte DNA synthesis and proliferation. However, when combined with L-ascorbic acid ( $10^{-6}$  M), these adrenergic agonists potentiated the hepatocyte DNA synthesis and proliferation induced by L-ascorbic acid. Western blot analysis showed that phenylephrine and metaproterenol did not potentiate L-ascorbic acid-induced insulin-like growth factor I receptor tyrosine kinase phosphorylation. In contrast, both phenylephrine and metaproterenol significantly potentiated L-ascorbic acid-induced extracellular-signal regulated kinase (ERK) phosphorylation within 3 min. Moreover, phorbol ester ( $10^{-7}$  M) and 8-bromo cAMP ( $10^{-7}$  M) also potentiated L-ascorbic acid-induced ERK2 phosphorylation. The effects of protein kinase C (PKC) and protein kinase A (PKA) activators were antagonized by PKC inhibitor GF109203X and PKA inhibitor H-89, respectively. These results suggest that indirect or direct activation of PKC and PKA represents a positive regulatory mechanism for potentiation of L-ascorbic acid-induced hepatocyte DNA synthesis and proliferation in primary cultures of adult rat hepatocytes.

## 1. Introduction

L-ascorbic acid, also known as vitamin C, is a nutritional supplement essential for preventing scurvy. L-ascorbic acid is reversibly oxidized in the body to dehydroascorbic acid, which retains full vitamin C activity. Vitamin C is an essential nutrient for the biosynthesis of collagen and L-carnitine, and for the conversion of dopamine to noradrenaline (Tajima and Pinnell, 1982; Li and Schellhorn, 2007). The liver is an important target for the antioxidant effects of vitamin C, and plays a role in body vitamin homeostasis. L-ascorbic acid and its derivatives can inhibit or stimulate the growth of normal and tumor cells, depending on cell type (Alcain and Buron, 1994; Belin et al., 2009; Koh et al., 1998; Ramírez-Farías et al., 2008; Yang et al., 2006). However, the cellular mechanisms of these actions are poorly understood.

The response of adult rat hepatocytes to L-ascorbic acid has not been investigated with respect to DNA synthesis and proliferation *in vitro*. More recently, we reported that L-ascorbic acid and its analogs are able to stimulate hepatocyte DNA synthesis and proliferation during short-term (e.g., 4 h) culture in primary cultures of adult rat hepatocytes by interacting with the insulin-like growth factor (IGF)-I receptor site and by activating the receptor tyrosine kinase/ extracellular-signal regulated kinase (ERK) pathway (Moteki et al., 2012). ERK1/2, also known as mitogen-activated protein kinase (p42/44 MAPK), is now known to be activated in response to a large number of mitogenic stimuli, and this enzyme is a key participant in the response to various growth factors and cytokines (Ginès et al., 1995; Moriuchi et al., 2001). In a previous

report, we showed that activation of extracellular-signal regulated kinase (ERK) isoform 2 (ERK2), but not ERK1, is involved in the L-ascorbic acid-induced DNA synthesis and proliferation in primary cultures of adult rat hepatocytes.

On the other hand, catecholamines (e.g., noradrenaline and its analogs) have been shown to be involved in the regulation of liver function (e.g., lipid metabolism, carbohydrate metabolism and cell growth). There are several types of catecholamine receptor,  $\beta_1$ - and  $\beta_2$ -receptors, that stimulate adenylate cyclase (AC) and increase cAMP, while  $\alpha_2$ -receptors inhibit this activity (Nakamura et al., 1984).  $\alpha_1$ -receptors are involved in phospholipase C activation and subsequent increases in inositolphosphate turnover and diacylglycerol production (Kimura and Ogihara, 1997a; Kimura and Ogihara, 1998; Dajani et al., 1990).

We have previously shown that  $\alpha$ - and  $\beta$ -adrenergic agonists significantly modulate hepatocyte DNA synthesis and proliferation in the presence of growth factors such as EGF, HGF, and IGF-I, -II (Kimura and Ogihara, 1997a, 1997b, 1998). However, there have been few studies on the adrenergic regulation of ERK1/2 phosphorylation induced by L-ascorbic acid in liver parenchymal cells. Therefore, to better understand the adrenergic regulation of the L-ascorbic acid-mediated signaling pathway, we examined whether  $\alpha_1$ -,  $\alpha_2$ - and  $\beta_2$ -adrenergic agonists can modulate L-ascorbic acid-induced ERK 1/2 isoform phosphorylation in the regulation of hepatocyte mitogenesis. The physiological significance of cross-talk between the L-ascorbic acid/IGF-I receptor-mediated pathway and  $\alpha_1$ -,  $\alpha_2$ -and  $\beta_2$ -adrenergic

receptor-mediated pathways in regulating hepatocyte proliferation is also discussed.

## **2. Materials and Methods**

### *2.1. Animals*

Male Wistar rats (weight, 200-220 g) were obtained from Saitama Experimental Animal Co. (Saitama, Japan). Rats were adapted to a light-, humidity- and temperature-controlled room over a minimum 3-day period prior to the start of experiments. Rats were fed a standard diet and given tap water *ad libitum*. Animals used in this study were handled in accordance with the “Guiding Principles for the Care and Use of Laboratory Animals”, as approved by the Ministry of Education, Culture, Sports, Science and Technology of Japan and the Guidelines for the Care and Use of Laboratory Animals of Josai University.

### *2.2. Hepatocyte isolation and culture*

The methods of hepatocyte isolation and culture were as described elsewhere (Kimura and Ogihara, 1997b). Briefly, rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (45 mg/kg). Two-step *in situ* collagenase perfusion was performed to facilitate disaggregation of the adult rat liver, as described previously (Seglen, 1975). The viability of hepatocytes consistently exceeded 96%, as determined by trypan blue exclusion assay. Unless otherwise indicated, isolated hepatocytes were plated onto 6-well collagen-coated plastic culture dishes (35 mm diameter; Iwaki Glass

Co., Tokyo, Japan) at a density of  $3.3 \times 10^4$  cells/cm<sup>2</sup> in minimum essential medium containing 5% bovine calf serum and  $10^{-10}$  M dexamethasone for 3 h in 5% CO<sub>2</sub> in air (Kimura et al., 2011). The medium was then changed, and cells were cultured in serum-free minimum essential medium containing various concentrations of L-ascorbic acid with or without adrenergic agonists or specific inhibitors of signal transducers. In this study, minimum essential medium was used in place of Williams' medium E because it does not contain L-ascorbic acid, vitamin E or vitamin K. L-ascorbic acid was buffered at pH 7.0 with sodium hydroxide and prepared fresh before each experiment.

### *2.3. Measurement of DNA synthesis*

Hepatocyte DNA synthesis was assessed by measuring [<sup>3</sup>H]-thymidine incorporation into acid-precipitable materials (Morley and Kingdon, 1972). After an initial attachment period of 3 h, hepatocytes were washed twice with serum-free minimum essential medium and cultured in medium containing L-ascorbic acid for a further 4 h. Cells were pulse-stimulated for 2 h with [<sup>3</sup>H]-thymidine (1.0 µCi/well) at 2 h following the addition of L-ascorbic acid. Incorporation of [<sup>3</sup>H]-thymidine into DNA was determined as described previously (Kimura and Ogihara, 1997b). Hepatocyte protein content was determined using a modified Lowry procedure (Lee and Paxman, 1972) with bovine serum albumin as a standard. Data are expressed as dpm/(h • mg cellular protein).

#### *2.4. Counting number of nuclei*

The number of nuclei rather than the number of cells was counted, as described previously, but with minor modifications (Nakamura et al., 1983). Briefly, primary cultured hepatocytes were washed twice with 2 ml of Dulbecco's phosphate-buffered saline (pH 7.4). Cells were then lysed by incubation in 0.25 ml of 0.1 M citric acid containing 0.1 % Triton X-100 for 30 min at 37°C. An equal volume of the nuclear suspension was mixed with 0.3% trypan blue in Dulbecco's phosphate-buffered saline (pH 7.4), and nuclei were counted in a hemocytometer.

#### *2.5. Determination of IGF-I receptor tyrosine kinase phosphorylation*

Tyrosine phosphorylation of the IGF-I receptor was identified by Western blotting using anti-phosphotyrosine antibody (Li et al., 1994). The phospho-IGF-I receptor antibody detects IGF-I receptor only when tyrosine 1161 in the carboxyl-terminal region is phosphorylated. This antibody does not cross-react with other tyrosine phosphorylated proteins. Briefly, hepatocytes were freshly isolated and seeded at a density of  $3.3 \times 10^4$  cells/cm<sup>2</sup> and cultured in minimal essential medium containing 5% newborn bovine serum. The medium was then aspirated and replaced, and cells were further cultured in serum-free minimal essential medium with or without L-ascorbic acid for various lengths of time. Cultured hepatocytes were washed once with ice-cold phosphate-buffered saline (pH 7.4) and then 0.2 ml of lysis buffer (20 mM Tris buffer, pH 7.5, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium

pyrophosphate, 1 mM sodium orthovanadate, 1 mM  $\beta$ -glycerophosphate and 1 mM phenylmethylsulfonyl fluoride) was added. Cell lysates were obtained by scraping the cells in lysis buffer, followed by sonication for 3 min. Cell lysates were centrifuged ( $3000 \times g$  for 3 min at  $4^{\circ}\text{C}$ ) to remove cellular debris, and were then denatured in boiling water for 5 min. For immunoblotting analysis, samples of the supernatant (30  $\mu\text{g}/\text{lane}$ ) were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) using a 7.5% polyacrylamide resolving gel, transferred to a polyvinylidene difluoride (PVDF) membrane, and immunoblotted with anti-phosphotyrosine antibody (Li et al., 1994). Blots were developed by enhanced chemiluminescence following incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies. The tyrosine kinase activity (autophosphorylation) of the phosphorylated p95-kDa protein (P-p95-kDa) was normalized against that of total p95-kDa protein. Quantification was performed by densitometry after development of the membrane with enhanced chemiluminescence reagent and exposure to Hyperfilm (Kodak, Japan). Densitometric analysis was performed using the NIH image program version 1.6 for Macintosh. Data were calculated in arbitrary units and are expressed as means  $\pm$  S.E.M. The autodiagram is representative of three experiments using different cell preparations.

## *2.6. Measurement of extracellular signal-regulated kinases 1 and 2 (ERK1/2) phosphorylation*



Phosphorylated ERK isoforms (pERK1; P-p44 MAPK and pERK2; P-p42 MAPK) were identified by Western blotting analysis using anti-phospho-ERK1/2 monoclonal antibody, as previously described (Towbin et al., 1979). Phosphorylated ERK1/2 phosphorylation was normalized against total ERK1/2 levels. Briefly, cultured hepatocytes were washed once with ice-cold phosphate-buffered saline (pH 7.4) and 0.2 ml of lysis buffer (10 mM Tris-HCl buffer (pH 7.4), 150 mM NaCl, 2 mM EGTA, 2 mM dithiothreitol, 1 mM sodium orthovanadate, 1 mM phenylmethylsulphonyl fluoride, 10 µg/ml leupeptin and 10 µg/ml aprotinin) was added, after which hepatocytes were harvested. After centrifugation at  $16,300 \times g$  for 30 min at 4°C, cell lysates were denatured in boiling water for 5 min. Supernatant samples (30 µg of protein) were subjected to SDS-PAGE using a 10% acrylamide resolving gel according to the method of Laemmli (Laemmli, 1970). After electrophoresis, proteins were transferred to Immobilon-P membranes.

For detection of phosphorylated extracellular-regulated kinase 1 and 2 (pERK1/2), membranes were immersed in Tris-buffered saline (pH 7.4) containing 1% bovine serum albumin. Membranes were then incubated with an antibody (1 µg/ml) against pERK1/2 and/or ERK1/2, and were washed as described previously (Towbin et al., 1979). Antibody binding was visualized by incubation with a horseradish peroxidase-conjugated donkey anti-rabbit IgG secondary antibody (1:3000 dilution), followed by enhanced chemiluminescence detection (ECL Kit; Amersham, UK). Densitometric analysis was performed using NIH Image version 1.68 for Macintosh.

Data were calculated in arbitrary units and are expressed as means  $\pm$  S.E.M.

Cytosolic protein in hepatocytes was quantified by modification of the Lowry procedure using bovine serum albumin as a standard (Lee and Paxman, 1972).

## 2.7. Materials

The following reagents were obtained from Sigma-Aldrich (St. Louis, MO): AG538 ( $\alpha$ -cyano-(3-methoxy-4-hydroxy-5-iodocinnamoyl)-(3', 4'-dihydroxyphenyl) ketone), aprotinin, 2,4-dideoxyadenosine, and dexamethasone. Rapamycin and PD98059 (2'-amino-3'-methoxyflavone) were obtained from R & D Systems (Minneapolis, MN). 8-bromo-cAMP, LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one hydrochloride), H-89 (N-[2-(p-bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide dihydrochloride), aphidicolin, metaproterenol hemisulfate, GF109203X hydrochloride (2-[1-(3-Dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)maleimide hydrochloride, phenylephrine hydrochloride, UK14304 (5-bromo-N-(4,5-dihydro-1H-imidazol-2-yl)-6-quinoxalinamine), and U-73122 (1-[6-[[17 $\beta$ ]-3-methoxyestra-1,3,5(10 $\beta$ )-trien-17-yl]amin]ohexyl]-1H-pyrrole-2,5-dione) were obtained from Enzo Life Sciences (Farmingdale, NY). 12-O-Tetradecanoylphorbol-13-acetate (phorbol ester, TPA) was obtained from Research Biochemicals Inc. (Natick, MA). Minimum essential medium and newborn calf serum were purchased from Flow Laboratories (Irvine, Scotland). Collagenase

(type II) was obtained from Worthington Biochemical Co. (Freehold, NJ). L-ascorbic acid was obtained from Wako Pure Chemicals Co. (Osaka, Japan).

Anti-phospho-ERK1/2 monoclonal antibody, anti-ERK1/2 monoclonal antibody were obtained from Cell Signaling Technology (Danvers, MA). Polyclonal anti-IGF-I receptor (phospho-Tyr1161) antibody and monoclonal anti-IGF-I receptor antibody were obtained from Applied Biochemical Materials Inc. (Richmond, BC). [Methyl-<sup>3</sup>H] thymidine (20 Ci/mmol) was purchased from DuPont-New England Nuclear (Boston, MA). All other reagents were of analytical grade.

## 2.8. Statistical Analysis

Group comparisons were made by ANOVA for unpaired data followed by post-hoc analysis using Dunnett's multiple comparison tests. Differences of  $P < 0.05$  were considered to be statistically significant.

## 3. Results

### 3.1. Potentiation of hepatocyte DNA synthesis and proliferation induced by L-ascorbic acid by metaproterenol, 8-bromo cAMP, phenylephrine and TPA

As shown in Figs. 1A and 1B, the  $\beta_2$ -adrenegic receptor agonist metaproterenol ( $10^{-6}$  M; Ogihara, 1996), 8-bromo cAMP ( $10^{-7}$  M), the  $\alpha_1$ -adrenegic receptor agonist phenylephrine ( $10^{-6}$  M; Kajiyama and Ui, 1998) and 12-O-tetradecanoylphorbol-13-acetate (phorbol ester, TPA;  $10^{-7}$  M; a direct PKC

activator; Smith et al., 1983) alone had no effect on hepatocyte DNA synthesis and proliferation. However, when combined, both phenylephrine and metaproterenol stimulated hepatocyte DNA synthesis (10.0-fold increase from baseline) and proliferation (1.4-fold increase from baseline) induced by L-ascorbic acid ( $10^{-6}$  M). The cell-permeable cAMP analog 8-bromo cAMP, a direct PKA activator (Derubertis and Zenser, 1976), and the direct PKC activator TPA also potentiated the L-ascorbic acid-induced hepatocyte DNA synthesis and proliferation. The  $\alpha_2$ -adrenergic receptor agonist UK14304 (Remaury et al., 1993) had no effect on the L-ascorbic acid-induced hepatocyte mitogenesis. However, the enhancing effects of metaproterenol on hepatocyte DNA synthesis and proliferation in the presence of L-ascorbic acid were inhibited by UK14304.

In order to confirm the notion that  $10^{-6}$  M L-ascorbic acid induces hepatocyte DNA synthesis and proliferation through receptor tyrosine kinase, PI3 kinase, ERK or the mammalian target of rapamycin (mTOR) phosphorylation, we investigated whether the IGF-I receptor tyrosine kinase inhibitor AG538 (Blum et al., 2000), the PI3 kinase inhibitor LY294002 (Vlahos et al., 1994), the MEK inhibitor PD98059 (Alessi et al., 1995) and the mTOR inhibitor rapamycin (Chung et al., 1992; Price et al., 1992) are able to inhibit L-ascorbic acid-induced hepatocyte DNA synthesis and proliferation in the presence or absence of metaproterenol and phenylephrine. Specific inhibitors of growth-related signal transducers, such as AG538 ( $10^{-7}$  M), LY294002 ( $10^{-7}$  M), PD98059 ( $10^{-6}$  M) and rapamycin (10 ng/ml) almost completely blocked L-ascorbic

acid-induced hepatocyte DNA synthesis and proliferation in the presence or absence of phenylephrine ( $10^{-6}$  M) and metaproterenol ( $10^{-6}$  M).

### *3.2. Effects of metaproterenol, 8-bromo cAMP, phenylephrine and TPA on L-ascorbic acid- induced IGF-I receptor tyrosine kinase phosphorylation*

In order to obtain further evidence for the cross-talk between the IGF-I receptor mediated-tyrosine kinase/ERK1/2 kinase pathway (ERK1/2 phosphorylation) and the  $\alpha_1$ -,  $\alpha_2$ - and  $\beta_2$ -adrenergic receptor-mediated pathway, we examined the effects of  $\alpha_1$ -,  $\alpha_2$ - and  $\beta_2$ -adrenergic receptor agonists on IGF-I receptor tyrosine kinase (Tyr1161) phosphorylation (p95-kDa protein) induced by L-ascorbic acid in the presence or absence of specific inhibitors of growth-related signal transducers. Figure 2 shows that L-ascorbic acid ( $10^{-6}$  M) caused an increase in tyrosine phosphorylation of a 95-kDa protein (IGF-I receptor tyrosine kinase) that peaked at about 3.0-fold (compared with control) 3 min after addition. Neither UK14304 ( $10^{-6}$  M), metaproterenol ( $10^{-6}$  M), 8-bromo cAMP ( $10^{-7}$  M), phenylephrine ( $10^{-6}$  M) nor TPA ( $10^{-7}$  M) had any effect on hepatocyte IGF-I receptor tyrosine kinase phosphorylation in the presence or absence of L-ascorbic acid ( $10^{-6}$  M) (Fig. 2). When L-ascorbic acid ( $10^{-6}$  M) was added in combination with AG538 ( $10^{-7}$  M), AG538 completely abolished the IGF-I-induced increase in receptor autophosphorylation (Tyr1161). In contrast, L-ascorbic acid-induced receptor tyrosine kinase phosphorylation was not abolished by LY294002 ( $10^{-7}$  M), PD98059 ( $10^{-6}$  M), rapamycin (10 ng/ml) treatment.

### *3.3. Time course and pattern of L-ascorbic acid stimulation of ERK isoform*

#### *phosphorylation, and their potentiation by $\beta_2$ -adrenergic agonist metaproterenol*

Figure 3A shows the typical pattern of phospho-ERK1/2 (pERK1/2) in isolated hepatocytes in culture as detected by Western blotting analysis after 1-60 min of culture. The phosphorylated ERK2 band (pERK2) was induced after only 1 min, and peaked (about 2.5-fold increase) between 3 and 5 min after addition of  $10^{-6}$  M L-ascorbic acid (Figs. 3A and 3B). The time course of the effects of a  $\beta_2$ -adrenergic agonist on ERK1/2 phosphorylation induced by  $10^{-6}$  M L-ascorbic acid was investigated using metaproterenol. Stimulation of hepatocytes with L-ascorbic acid in the presence of metaproterenol ( $10^{-6}$  M) induced a more rapid and significant increase in ERK2 phosphorylation than stimulation with L-ascorbic acid alone, reaching a peak at 5 min after addition (about 3.5-fold increase from baseline) and rapidly declining to basal levels within 30 min (Figs. 3A and 3B). In contrast, metaproterenol alone did not significantly stimulate ERK2 phosphorylation (data not shown). In addition, metaproterenol in the presence of L-ascorbic acid did not significantly stimulate ERK1 phosphorylation (Figs. 3A and 3C). These data indicate that metaproterenol ( $10^{-6}$  M) specifically potentiated L-ascorbic acid-induced ERK2 phosphorylation.

### *3.4. Effects of specific inhibitors of signal transducers on metaproterenol or 8-bromo cAMP-induced ERK1/2 isoform phosphorylation in presence of L-ascorbic acid*

In order to investigate the mechanisms by which the  $\beta_2$ -adrenergic receptor/protein

kinase A (PKA) pathway potentiates L-ascorbic acid induction of ERK2 phosphorylation, we examined the effects of metaproterenol (indirect PKA stimulator) and the cell-permeable cAMP analog 8-bromo cAMP (direct PKA stimulator) on L-ascorbic acid-induced ERK2 phosphorylation (Figs. 4 and 5). The dose-dependent potentiation of ERK2 phosphorylation induced by either metaproterenol (Fig. 4) or 8-bromo cAMP (Fig. 5) was confirmed in the presence of L-ascorbic acid, and was almost completely reduced to control levels by AG538 ( $10^{-7}$  M) LY294002 ( $10^{-7}$  M) and PD98059 ( $10^{-6}$  M), but not by rapamycin (Figs. 4 and 5). In addition, the  $\alpha_2$ -adrenergic agonist UK14304 (indirect adenylate cyclase (AC) inhibitor) dose-dependently inhibited the metaproterenol-induced potentiation of ERK2 phosphorylation in the presence of L-ascorbic acid (Fig. 4), but not 8-bromo cAMP-induced potentiation (Fig. 5). Furthermore, pretreatment of hepatocytes with the direct AC inhibitor 2,4-dideoxyadenosine ( $10^{-6}$  M; Holgate et al., 1980) blocked the potentiating effect of metaproterenol on the phosphorylation of ERK2 in the presence of L-ascorbic acid, but not the effects of 8-bromo cAMP. In contrast, pretreatment of hepatocytes with the PKA inhibitor H-89 ( $10^{-7}$  M; Zuscik et al., 1994) blocked the potentiating effects of metaproterenol and 8-bromo cAMP on the phosphorylation of ERK2 in the presence of L-ascorbic acid. UK14304 ( $10^{-7}/10^{-6}$  M), metaproterenol ( $10^{-6}$  M) or 8-bromo cAMP ( $10^{-7}$  M) on its own had no significant effect on the phosphorylation of ERK1 or ERK2 (Figs. 4 and 5).

### *3.5. Time course and pattern of L-ascorbic acid stimulation of ERK isoform*

#### *phosphorylation, and their potentiation by $\alpha_1$ -adrenergic agonist phenylephrine*

The effects of an  $\alpha_1$ -adrenergic agonist on the induction of ERK phosphorylation by  $10^{-6}$  M L-ascorbic acid were investigated using phenylephrine. L-ascorbic acid in the presence of phenylephrine ( $10^{-6}$  M; an indirect PKC activator) caused a more rapid and significant increase in ERK2 phosphorylation than L-ascorbic acid alone. ERK2 phosphorylation peaked at 3 min (about 3.0-fold increase from baseline) after addition and rapidly decreased to basal levels within 30 min (Figs. 6A and 6B). In contrast, phenylephrine alone did not significantly stimulate ERK2 phosphorylation (data not shown). Phenylephrine ( $10^{-6}$  M) in the presence of  $10^{-6}$  M L-ascorbic acid did not significantly stimulate ERK1 phosphorylation (Fig. 6C). The present findings indicate that phenylephrine potentiates L-ascorbic acid-induced ERK2 phosphorylation.

### *3.6. Effects of specific inhibitors of signal transducers on phenylephrine- or*

#### *TPA-Induced ERK isoform phosphorylation in presence of L-ascorbic acid*

The effects of an  $\alpha_1$ -adrenergic agonist on induction of ERK1/2 phosphorylation by  $10^{-6}$  M L-ascorbic acid were investigated using phenylephrine ( $10^{-6}$  M) and a direct PKC activator TPA ( $10^{-7}$  M). When hepatocytes were stimulated with L-ascorbic acid in the presence of phenylephrine ( $10^{-6}$  M), or TPA ( $10^{-7}$  M), significant potentiation of ERK2 phosphorylation was also observed when compared with L-ascorbic acid ( $10^{-6}$  M) alone (Fig. 7). L-ascorbic acid-induced ERK2 phosphorylation in the presence of



phenylephrine ( $10^{-6}$  M) was also completely blocked by AG538, LY294002 and PD98059, but was not blocked by rapamycin. Pretreatment of hepatocytes with the phospholipase C (PLC) inhibitor U-73122 ( $10^{-6}$  M; Thompson et al., 1991), or the PKC inhibitor GF109203X ( $10^{-7}$  M; Toullec et al., 1991) blocked the L-ascorbic acid-induced phosphorylation of ERK2 in the presence of phenylephrine ( $10^{-6}$  M). However, pretreatment of hepatocytes with the phospholipase C (PLC) inhibitor U-73122 ( $10^{-6}$  M) did not block the L-ascorbic acid-induced phosphorylation of ERK2 in the presence of TPA ( $10^{-7}$  M), while the PKC inhibitor GF109203X ( $10^{-7}$  M) did. Phenylephrine ( $10^{-6}$  M), TPA ( $10^{-7}$  M), U-73122 ( $10^{-6}$  M) or GF109203X alone had no significant effects on the phosphorylation of ERK1 or ERK2 (Fig. 7).

#### **4. Discussion**

As shown in Figs. 1A and 1B, we found that both the  $\alpha_1$ -adrenergic agonist phenylephrine and the  $\beta_2$ -adrenergic agonist metaproterenol potentiated the hepatocyte DNA synthesis and proliferation induced by L-ascorbic acid ( $10^{-6}$  M). The potentiating effects of phenylephrine and metaproterenol on the L-ascorbic acid-induced hepatocyte DNA synthesis and proliferation were almost completely blocked by inhibitors of growth-related signal transducers, such as AG538 ( $10^{-7}$  M), LY294002 ( $10^{-7}$  M), PD98059 ( $10^{-6}$  M) and rapamycin (10 ng/mL) (Fig. 1). These results suggest that there is cross-talk between  $\alpha_1$ - and  $\beta_2$ -adrenergic receptor-mediated pathway and IGF-I receptor-mediated pathway in the regulation of L-ascorbic acid-induced hepatocyte

mitogenesis. In addition, the enhancing effects of  $\beta_2$ -adrenergic receptor agonist metaproterenol on hepatocyte DNA synthesis and proliferation in the presence of L-ascorbic acid were inhibited by  $\alpha_2$ -adrenergic receptor agonists UK14304 (Fig.1).

In order to obtain further evidence for the cross-talk between L-ascorbic acid-induced IGF-I receptor tyrosine kinase/ERK1/2 kinase activation (ERK1/2 phosphorylation) and  $\alpha_1$ - or  $\beta_2$ -adrenergic receptor activation, we examined the effects of specific inhibitors or activators of growth-related signal transducers and  $\alpha_2$ - and  $\beta_2$ -adrenergic receptor agonists on IGF-I receptor tyrosine kinase phosphorylation. As shown in Figure 2, L-ascorbic acid ( $10^{-6}$  M) caused an increase in the IGF-I receptor tyrosine phosphorylation of a 95-kDa protein that peaked 3 min after addition. When L-ascorbic acid ( $10^{-6}$  M) was added in combination with AG538 ( $10^{-7}$  M), AG538 completely abolished the L-ascorbic acid-induced increase in receptor autophosphorylation (Tyr1161). In contrast, L-ascorbic acid-induced IGF-I receptor tyrosine kinase activation was not abolished by LY294002 ( $10^{-7}$  M), PD98059 ( $10^{-6}$  M), rapamycin (10 ng/ml) treatment. These results indicate that IGF-I receptor tyrosine kinase phosphorylation is specifically activated by L-ascorbic acid.

The present results are also consistent with the reports that ERK1/2 and/or mTOR/p70S6K lie downstream of the IGF-I receptor tyrosine kinase in primary cultures of adult rat hepatocytes (Moteki et al., 2012; Kimura and Ogihara, 1998; Blum et al., 2000; Alexia et al., 2006). As neither the  $\alpha_1$ -adrenergic receptor agonist phenylephrine ( $10^{-6}$  M), the diacylglycerol derivative TPA ( $10^{-7}$  M),  $\beta_2$ -adrenergic

receptor agonist metaproterenol ( $10^{-6}$  M) nor the cell-permeable cAMP analog 8-bromo cAMP ( $10^{-7}$  M) had any effect on the L-ascorbic acid-induced receptor tyrosine kinase phosphorylation (Fig. 2), the results demonstrate that  $\alpha_1$ - or  $\beta_2$ -adrenergic receptor stimulation do not directly interact with the IGF-I receptor stimulation to induce receptor tyrosine kinase phosphorylation.

It has been reported that ERK plays an important role in the proliferation of hepatocytes and other cells induced by growth factors and cytokines (Ginès et al., 1995; Moriuchi et al., 2001; Alexia et al., 2006; Mebratu and Tesfaigzi, 2009; Katz et al., 2007). The proliferative pathway by which L-ascorbic acid activates the IGF-I receptor tyrosine kinase (p95 kDa)/ ERK1/2 pathway (MAPK cascade) has been described in primary cultures of hepatocytes (Moteki et al., 2012). However, there have been very few studies regarding  $\alpha$ - and  $\beta$ -adrenergic receptor-mediated regulation of ERK1/2 phosphorylation induced by L-ascorbic acid in primary cultured hepatocytes. Therefore, we examined whether  $\alpha_2$ - and  $\beta_2$ -adrenergic agonists modulate the L-ascorbic acid-induced changes in ERK2 phosphorylation.

As shown in Fig. 3, we found that L-ascorbic acid-induced hepatocyte ERK2 phosphorylation was enhanced by metaproterenol ( $10^{-6}$  M), an indirect adenylate cyclase (AC) activator, after 5 min of culture (Figs. 3 and 4). In contrast, metaproterenol alone had no significant effects on hepatocyte ERK1/2 phosphorylation in the absence of L-ascorbic acid (Fig. 4). As shown in a recent report, L-ascorbic acid ( $10^{-6}$  M) at a concentration that induces hepatocyte DNA synthesis and proliferation

rapidly stimulated ERK2, but not ERK1 phosphorylation (Moteki et al., 2012). Frémin et al. showed that continued expression of cyclin-D1 in the G<sub>0</sub>/G<sub>1</sub> phase is dependent on ERK2 expression by L-ascorbic acid, and that the ablation of ERK1 influences neither the proliferation capacity of mice hepatocytes in this regulation, *in vivo* and *in vitro*, nor the expression pattern of cyclin-D1 (Frémin et al., 2007). In agreement with a recent report, our data indicate that ERK2 plays a key role in hepatocyte proliferation (Frémin et al., 2007; Frémin et al., 2012). Moreover, L-ascorbic acid induction of ERK2 phosphorylation in hepatocytes was almost completely blocked by specific inhibitors of signal transducers, such as the IGF-I receptor tyrosine kinase inhibitor AG538, PI3 kinase inhibitor LY294002, and the MEK inhibitor PD98059, but not by rapamycin (mTOR inhibitor), thus suggesting that mTOR lies downstream of MEK (Moteki et al., 2012).

The enhancing effects of metaproterenol in the presence of L-ascorbic acid were inhibited by  $\alpha_2$ -adrenergic receptor agonist UK14304 ( $10^{-6}$  M; indirect AC inhibitor which decreases cAMP), 2,4-dideoxyadenosine ( $10^{-6}$  M; direct AC inhibitor) and the PKA inhibitor H-89, thus suggesting the involvement of PKA in the potentiating effects of metaproterenol (Fig. 4). The involvement of PKA in the effects of metaproterenol is supported by our previous results, which indicated that metaproterenol-induced potentiation of hepatocyte DNA synthesis and proliferation in the presence of L-ascorbic acid ( $10^{-6}$  M) was completely inhibited by a specific PKA inhibitor, H-89 (data not shown).

However, the role of the second messenger cAMP in the control of hepatocyte DNA synthesis and proliferation remains uncertain (Brønstad et al., 1983; Liu et al., 2005). cAMP can either stimulate or inhibit DNA synthesis, depending on culture conditions (Kimura and Ogihara, 1998; Brønstad et al., 1983; Liu et al., 2005). We demonstrated that the cell-permeable cAMP analog 8-bromo cAMP dose-dependently enhances hepatocyte proliferation and ERK2 phosphorylation induced by L-ascorbic acid (Figs. 1 and 5). These results indicate that there is cross-talk between the  $\beta_2$ -adrenergic receptor/AC/cAMP pathway and the IGF-I receptor/ERK pathway, which potentiates hepatocyte mitogenesis. Because both metaproterenol- and 8-bromo cAMP-induced potentiation of ERK2 phosphorylation in the presence of L-ascorbic acid are completely inhibited by AG538, LY294002 and PD98059, but not by rapamycin, the cross-talk signals mediated by the  $\beta_2$ -adrenergic pathway may converge upstream of ERK2.

We examined whether an  $\alpha_1$ -adrenergic agonist modulates the L-ascorbic acid-induced changes in ERK2 phosphorylation. As shown in Figs. 6 and 7, while an  $\alpha_1$ -adrenergic agonist phenylephrine alone had no effect on hepatocyte ERK phosphorylation in the absence of L-ascorbic acid, L-ascorbic acid-induced ERK2 phosphorylation was enhanced by phenylephrine. The enhancing effects of phenylephrine in the presence of L-ascorbic acid was inhibited by the PKC inhibitor, GF109203X ( $10^{-7}$  M), thus suggesting the involvement of PKC in the potentiating effects of phenylephrine (Fig. 7). The involvement of PKC in the phenylephrine effect

is supported by our previous results, indicating that phenylephrine-induced potentiation of hepatocyte DNA synthesis and proliferation in the presence of L-ascorbic acid ( $10^{-6}$  M) was completely inhibited by a specific PKC inhibitor, GF109203X (data not shown).

However, the roles of second messengers, such as  $IP_3$  and diacylglycerol, in the control of hepatocyte DNA synthesis and proliferation remain uncertain (Berridge, 1993). Phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA;  $10^{-7}$  M), a direct activator of PKC, can either directly or indirectly stimulate DNA synthesis depending on culture conditions (Kimura and Ogihara, 1998; Ginès et al., 1995; O'Brien et al., 1979). In addition,  $\alpha_1$ -adrenergic receptor-mediated signals may interact with upstream signal transducers, such as the MEK, raf or ras pathways (Ramirez et al., 1997; Xiao et al., 2001), and have a positive influence normal liver growth and liver regeneration after partial hepatectomy in vivo (Huh et al., 2004; Beyer et al., 2008). As shown in Fig.7, both phenylephrine (indirect PLC/PKC activator)- and TPA-induced potentiation of ERK2 phosphorylation in the presence of L-ascorbic acid were completely inhibited by AG538, LY294002 and PD98059, but not by rapamycin (Fig. 7). Thus, the  $\alpha_1$ -adrenergic receptor/ PLC/ PKC pathway may interact with upstream elements such as raf or ras (Ginès et al., 1995; Ramirez et al., 1997; Xiao et al., 2001).

Based on the present data, a possible scheme for cross-talk between the L-ascorbic acid-targeted IGF-I receptor/ERK2 pathway and the  $\alpha_1$ -adrenergic receptor/PLC/PKC pathway and/or  $\beta_2$ -adrenergic receptor/AC/PKA pathway is summarized in Fig. 8. The

cross-talk signals of the  $\alpha_1$ - and  $\beta_2$ -adrenergic pathway may influence growth-related signal transducers such as Ras, Raf and MEK that lie downstream of the IGF-I receptor tyrosine kinase, but upstream of ERK (Ginès et al., 1995; Ramirez et al., 1997; Xiao et al., 2001; Pollak et al., 2004). More detailed mechanisms of cross-talk between the L-ascorbic acid-signaling pathway and the  $\alpha_1$ -receptor/PLC/PKC and/or  $\beta_2$ -adrenergic receptor/AC/PKA pathway remain to be explored.

In conclusion, the present study demonstrates that the cross-talk signals by extracellular  $\alpha_1$ - and  $\beta_2$ -adrenoceptor agonists, such as phenylephrine and metaproterenol, potentiate L-ascorbic acid-induced ERK2 phosphorylation in primary cultured adult rat hepatocytes. Furthermore, the cross-talk signaling may converge between downstream of L-ascorbic acid-targeted IGF-I receptor tyrosine kinase, and upstream of MEK/ERK and mTOR/p70 S6K (Fig. 8). The present findings support the notion that endogenous catecholamine-induced potentiation of hepatocyte DNA synthesis and proliferation in the presence of L-ascorbic acid play an important role in the activation of the ERK cascade during liver regeneration after partial hepatectomy or recovery from liver necrosis caused by toxic chemicals *in vivo* (Ramírez-Farías et al., 2008).

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## Figure legends

**Fig. 1.** Potentiation by metaproterenol, 8-bromo cAMP, phenylephrine and TPA of hepatocyte DNA synthesis and proliferation induced by L-ascorbic acid.

Freshly isolated hepatocytes were plated at a density of  $3.3 \times 10^4$  cells/cm<sup>2</sup> and cultured as described in Materials and Methods. After a medium change, hepatocytes were cultured with L-ascorbic acid ( $10^{-6}$  M), with or without the indicated specific inhibitors or activators of signal transducers for 4 h.

Concentrations were as follows: UK14304,  $10^{-6}$  M; metaproterenol,  $10^{-6}$  M; 8-bromo cAMP,  $10^{-7}$  M; phenylephrine,  $10^{-6}$  M; TPA,  $10^{-7}$  M; AG538,  $10^{-7}$  M; LY294002,  $10^{-7}$  M; PD98059,  $10^{-6}$  M; and rapamycin, 10 ng/ml.

The rate of hepatocyte DNA synthesis is expressed as dpm/(h · mg cellular protein) (A). Hepatocyte proliferation is expressed as the percent increase in total number of nuclei, as compared with control culture (B). Results are expressed as means  $\pm$  S.E.M. of three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$  vs. respective controls. # $P < 0.05$ , ## $P < 0.01$  vs. L-ascorbic acid plus metaproterenol ( $10^{-6}$  M)-treated controls (L-ascorbic acid+metaproterenol). \$ $P < 0.05$ , \$\$ $P < 0.01$  vs. L-ascorbic acid plus phenylephrine ( $10^{-6}$  M)-treated controls (L-ascorbic acid + phenylephrine).

**Fig. 2.** Effects of metaproterenol, 8-bromo cAMP, phenylephrine and TPA on L-ascorbic acid-induced IGF-I receptor tyrosine kinase phosphorylation.

Freshly isolated hepatocytes were plated at a density of  $3.3 \times 10^4$  cells/cm<sup>2</sup> and

cultured as described in Materials and Methods. After a medium change, hepatocytes were cultured with L-ascorbic acid ( $10^{-6}$  M) with or without the indicated specific inhibitors or activators of signal transducers for 3 min.

Concentrations were as follows: UK14304,  $10^{-6}$  M; metaproterenol,  $10^{-6}$  M; 8-bromo cAMP,  $10^{-7}$  M; phenylephrine,  $10^{-6}$  M; TPA,  $10^{-7}$  M; AG538,  $10^{-7}$  M; LY294002,  $10^{-7}$  M; PD98059,  $10^{-6}$  M; and rapamycin, 10 ng/ml.

Phosphorylated IGF-I receptor tyrosine kinase (P-p95 kDa) (upper bands) and total receptor tyrosine kinase protein (p95 kDa) were determined by Western blotting using an anti-phospho-IGF-I receptor (Tyr1161) antibody, as described in Materials and Methods. Typical Western blot images are indicated on the top of the figure. Results are expressed as means  $\pm$  S.E.M. of three independent experiments. \* $P < 0.05$  vs. respective controls. <sup>#</sup> $P < 0.05$  vs. L-ascorbic acid plus metaproterenol ( $10^{-6}$  M)-treated controls (L-ascorbic acid + metaproterenol). <sup>\$</sup> $P < 0.05$  vs. L-ascorbic acid plus phenylephrine ( $10^{-6}$  M)-treated controls (L-ascorbic acid + phenylephrine).

**Fig. 3.** Time course and patterns of L-ascorbic acid stimulation of ERK isoform phosphorylation, and their potentiation by metaproterenol.

Isolated hepatocytes were cultured for 3 h, and were then washed and incubated in either the absence (control; C: medium alone) or presence of  $10^{-6}$  M L-ascorbic acid (AA), or  $10^{-6}$  M L-ascorbic acid with  $10^{-6}$  M metaproterenol (A+M) for the indicated times (min) at 37°C. Phosphorylated ERK isoforms (pERK1/2) were identified by

Western blotting using an anti-pERK antibody, as described in Materials and Methods. The intensity of the  $M_r$  44-kDa and 42-kDa bands, corresponding to pERK1 and pERK2, respectively, was normalized against total ERK (ERK1/2). A: typical Western blot images; B: time-course of ERK2 phosphorylation; C: time-course of ERK1 phosphorylation. Results are expressed as a percentage of the respective control value (mean  $\pm$  SEM of three experiments). \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001 vs. respective controls (medium alone).

**Fig. 4.** Effects of specific inhibitors of signal transducers on metaproterenol-induced ERK1/2 isoform phosphorylation in presence of L-ascorbic acid.

Hepatocytes were stimulated for 5 min with or without L-ascorbic acid ( $10^{-6}$  M) in the presence or absence of metaproterenol ( $10^{-8}$  to  $10^{-6}$  M) and in the presence or absence of specific signal transducer inhibitors. Concentrations are as follows: AG538 ( $10^{-7}$  M), LY294002 ( $10^{-7}$  M), PD98059 ( $10^{-6}$  M), rapamycin (10 ng/ml), H-89 ( $10^{-7}$  M) or 2,4,-dideoxyadenosine ( $10^{-6}$  M) and/or  $\alpha_2$ -adrenegic receptor agonist UK14304 ( $10^{-7}$ ,  $10^{-6}$  M). Phosphorylated ERK isoforms (pERK1 and pERK2) were determined by Western blotting using an anti-phospho-ERK1/2 antibody, as described in the legend to Fig. 3. Typical Western blot images are indicated on the top of the figure. Results are expressed as a percentage of the respective control value (means  $\pm$  SEM of three experiments). \* $P$ <0.05, \*\* $P$ <0.01 vs. respective L-ascorbic acid-treated controls (L-ascorbic acid alone), # $P$ <0.05, ## $P$ <0.01 vs. L-ascorbic acid plus metaproterenol

( $10^{-6}$  M)-treated controls.

**Fig. 5.** Effects of specific inhibitors of signal transducers on 8-bromo cAMP-induced ERK isoform phosphorylation in presence of L-ascorbic acid.

Hepatocytes were stimulated for 5 min with or without L-ascorbic acid ( $10^{-6}$  M) in the presence or absence of 8-bromo cAMP ( $10^{-9}$ - $10^{-7}$  M) and in the presence or absence of specific signal transducer inhibitors. Concentrations are as follows: AG538 ( $10^{-7}$  M), LY294002 ( $10^{-7}$  M), PD98059 ( $10^{-6}$  M), rapamycin (10 ng/ml), 2,4-dideoxyadenosine ( $10^{-6}$  M) or H-89 ( $10^{-7}$  M) and/or  $\alpha_2$ -adrenegic receptor agonist UK14304 ( $10^{-7}$ / $10^{-6}$  M). Phosphorylated ERK1/2 isoforms (pERK1/2) were determined by Western blotting using an anti-phospho-ERK1/2 antibody, as described in the legend to Fig. 3. Typical Western blot images are indicated on the top of the figure. Results are expressed as a percentage of the respective control value (mean  $\pm$  SEM of three experiments). \* $P$ <0.05, \*\* $P$ <0.01 vs. respective L-ascorbic acid -treated controls (L-ascorbic acid alone). # $P$ <0.05, ## $P$ <0.01 vs. L-ascorbic acid plus 8-bromo cAMP ( $10^{-7}$  M)-treated controls.

**Fig. 6.** Time course and patterns of L-ascorbic acid stimulation of ERK isoform phosphorylation, and their potentiation by  $\alpha_1$ -adrenergic agonist phenylephrine.

Isolated hepatocytes were cultured for 3 h, then washed and incubated in either the absence (control; C: medium alone) or presence of  $10^{-6}$  M L-ascorbic acid (AA) alone,

or  $10^{-6}$  M L-ascorbic acid with  $10^{-6}$  M phenylephrine (A+P) for the indicated times (min) at 37°C. Phosphorylated ERK isoforms (pERK1/2) were identified by Western blotting using an anti-pERK antibody, as described in the legend to Fig. 3. The intensity of the  $M_r$  44-kDa and 42-kDa bands, corresponding to pERK1 and pERK2, respectively, was normalized to total ERK (ERK1/2). A: Typical Western blot images; B: time-course of ERK2 phosphorylation; C: time-course of ERK1 phosphorylation. Results are expressed as a percentage of the respective control value (means  $\pm$  SEM of three experiments). \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001 vs. respective controls (medium alone).

**Fig. 7.** Effects of specific inhibitors of signal transducers on phenylephrine- or TPA-induced ERK isoform phosphorylation in presence of L-ascorbic acid.

Hepatocytes were stimulated for 5 min with or without L-ascorbic acid ( $10^{-6}$  M) in the presence or absence of phenylephrine ( $10^{-8}$ - $10^{-6}$  M), and/or TPA ( $10^{-7}$  M) and in the presence or absence of specific inhibitors of the signal transducers. The concentrations are as follows: AG538 ( $10^{-7}$  M), LY294002 ( $10^{-7}$  M), PD98059 ( $10^{-6}$  M), rapamycin (10 ng/ml), U-73122 ( $10^{-6}$  M) and GF109203X ( $10^{-7}$  M). Phosphorylated ERK1/2 isoforms (pERK1/2) were determined by Western blotting using an anti-phospho-ERK1/2 antibody, as described in the legend to Fig. 3. Typical Western blot images are indicated on the top of the figure. Results are expressed as a percentage of respective control values (means  $\pm$  SEM of three experiments). \* $P$ <0.05, \*\* $P$ <0.01

vs. respective L-ascorbic acid-treated controls (L-ascorbic acid alone),  $^{\$}P<0.05$ ,  
 $^{\$\$}P<0.01$  vs. L-ascorbic acid plus phenylephrine ( $10^{-6}$  M)-treated  
controls,  $^{\text{!}}P<0.05$ ,  $^{\text{!!}}P<0.01$  vs. L-ascorbic acid plus TPA ( $10^{-7}$  M)-treated controls.

**Fig. 8.** Possible scheme for cross-talk between L-ascorbic acid-targeted IGF-I  
receptor/ERK2 pathway and  $\alpha_1$ -,  $\alpha_2$ - or  $\beta_2$ -adrenergic receptor-mediated pathways.

Abbreviations: IGF-I, insulin-like growth factor I; RTK, receptor tyrosine kinase;  
PI3K, phosphatidylinositol-3 kinase; MEK, MAP kinase kinase (upstream signal  
transducer of ERK); ERK2, extracellular-signal regulated kinase 2 (p42 MAPK);  
mTOR, mammalian target of rapamycin; p70S6K, p70 ribosomal protein S6 kinase  
(downstream signal transducer of mTOR); AC, adenylate cyclase; PKA, protein kinase  
A; PIP<sub>2</sub>, phosphatidylinositol 4,5-biphosphate; PLC, phospholipase C; PKC, protein  
kinase A;  $\rightarrow$  or  $\oplus$ , stimulation;  $\text{---|}$  or  $\ominus$ , inhibition.

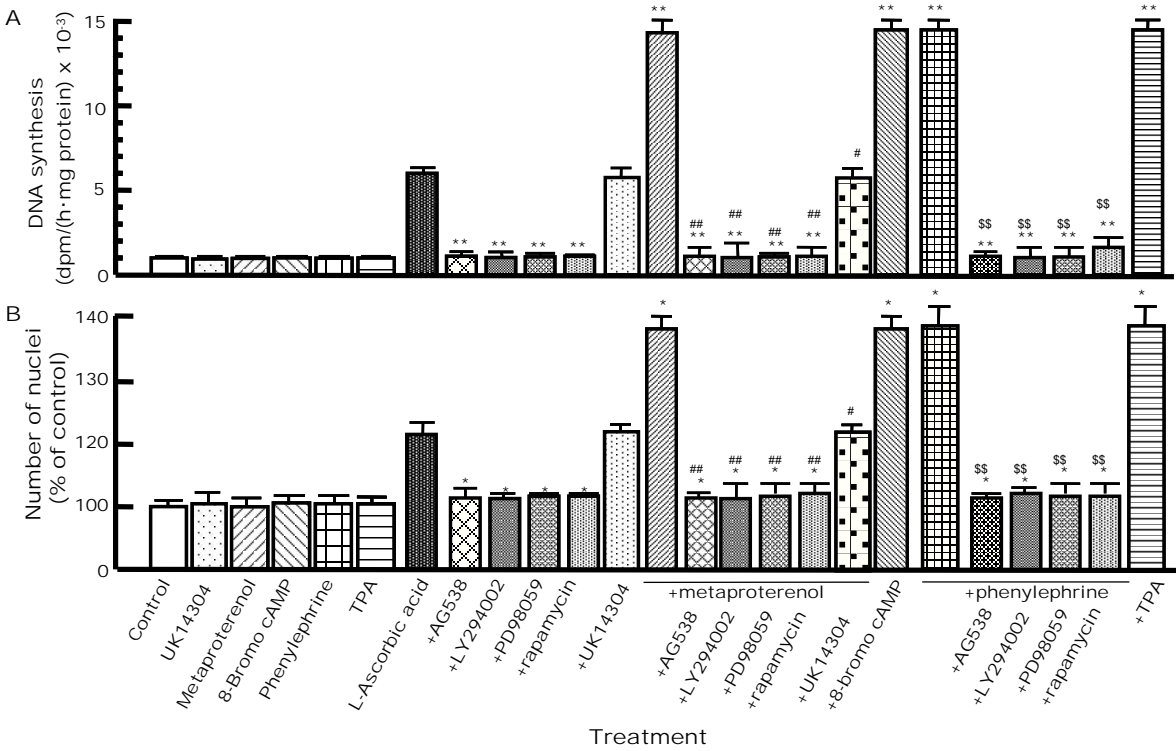
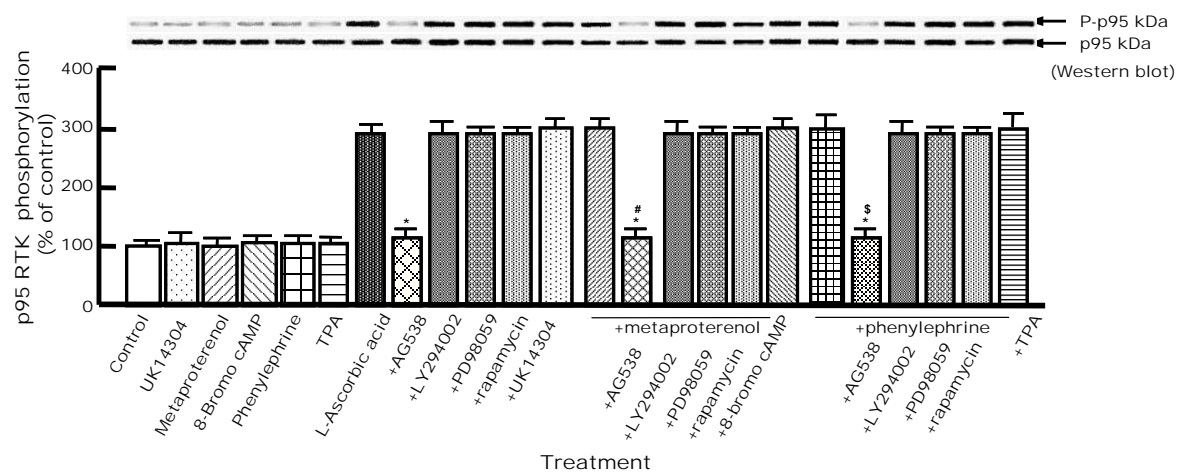


Fig.1

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**Fig.2**

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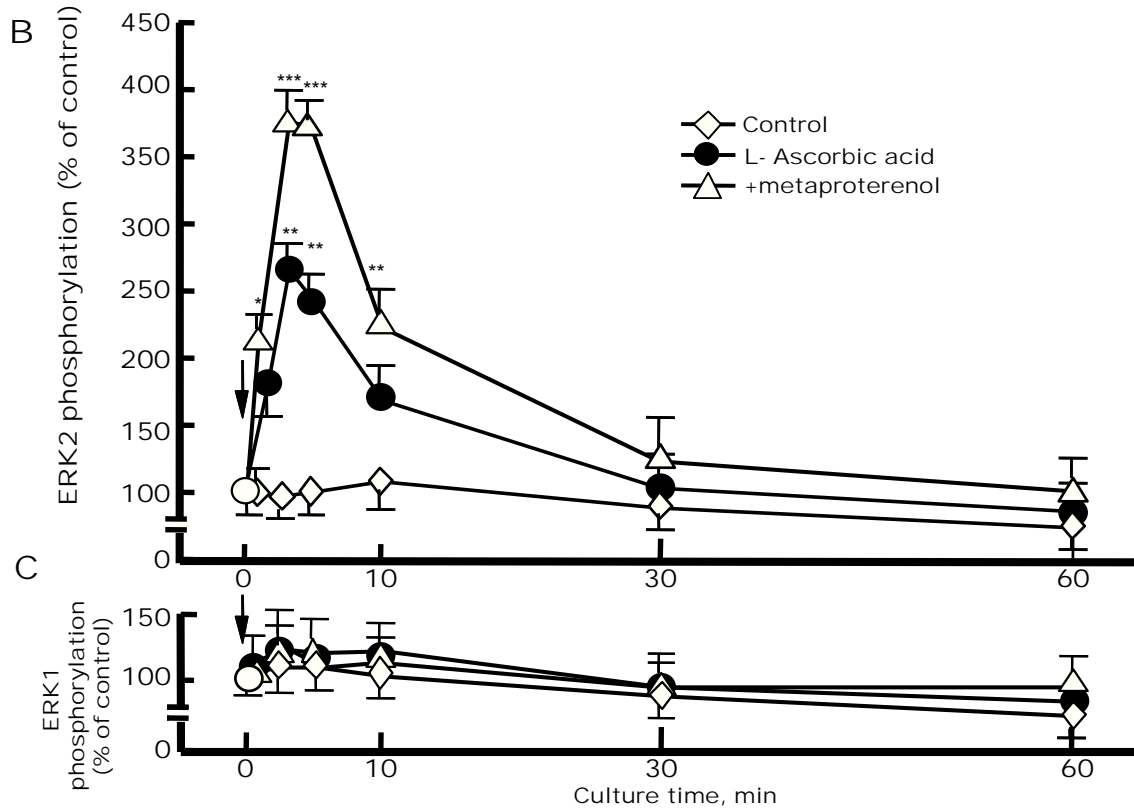
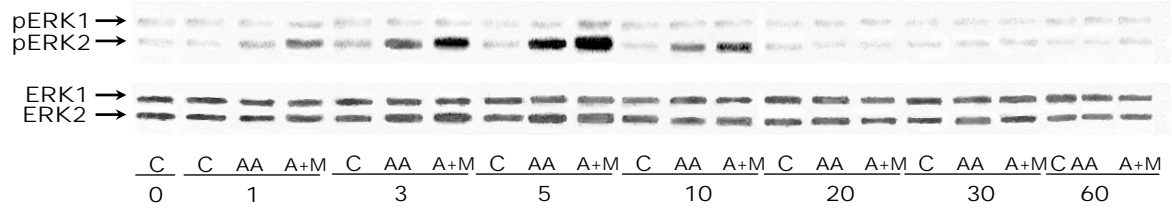
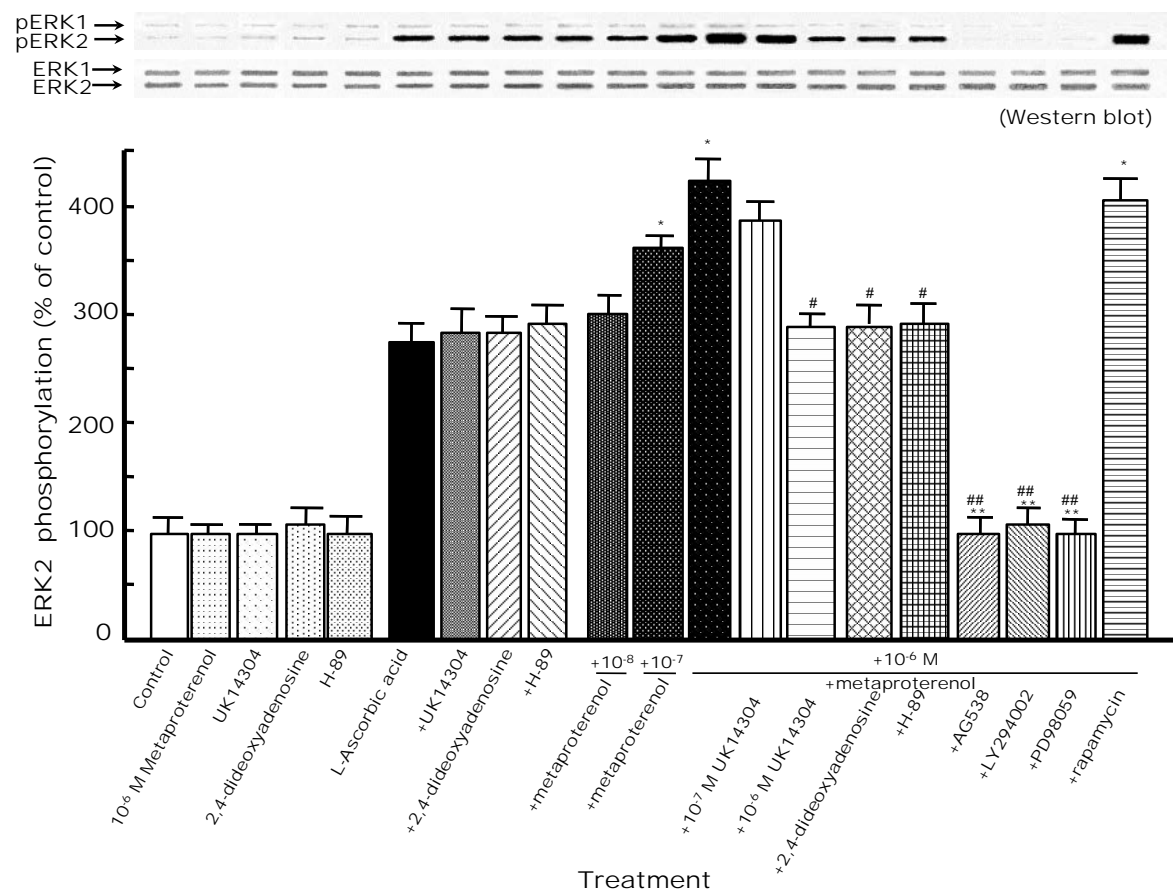


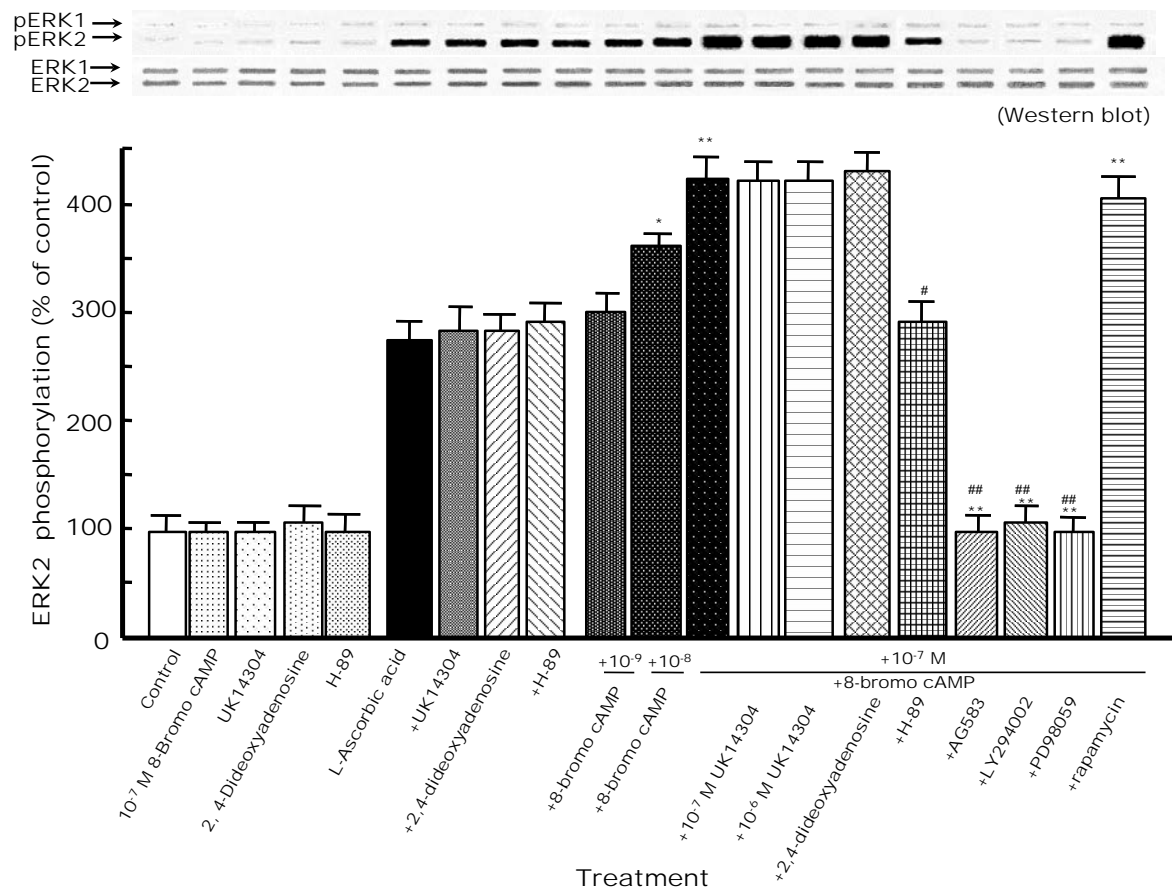
Fig.3

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**Fig.4**

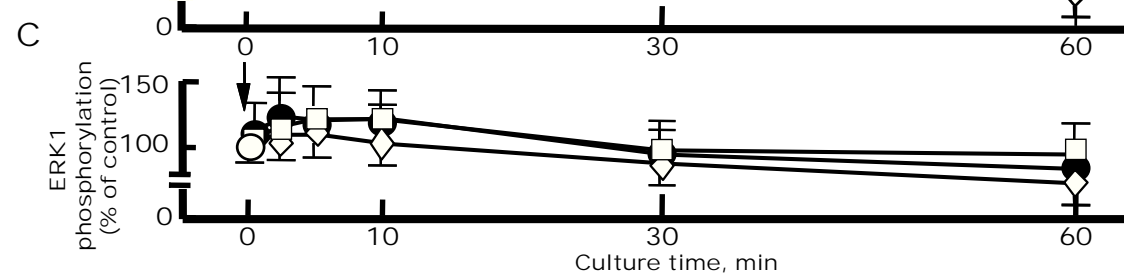
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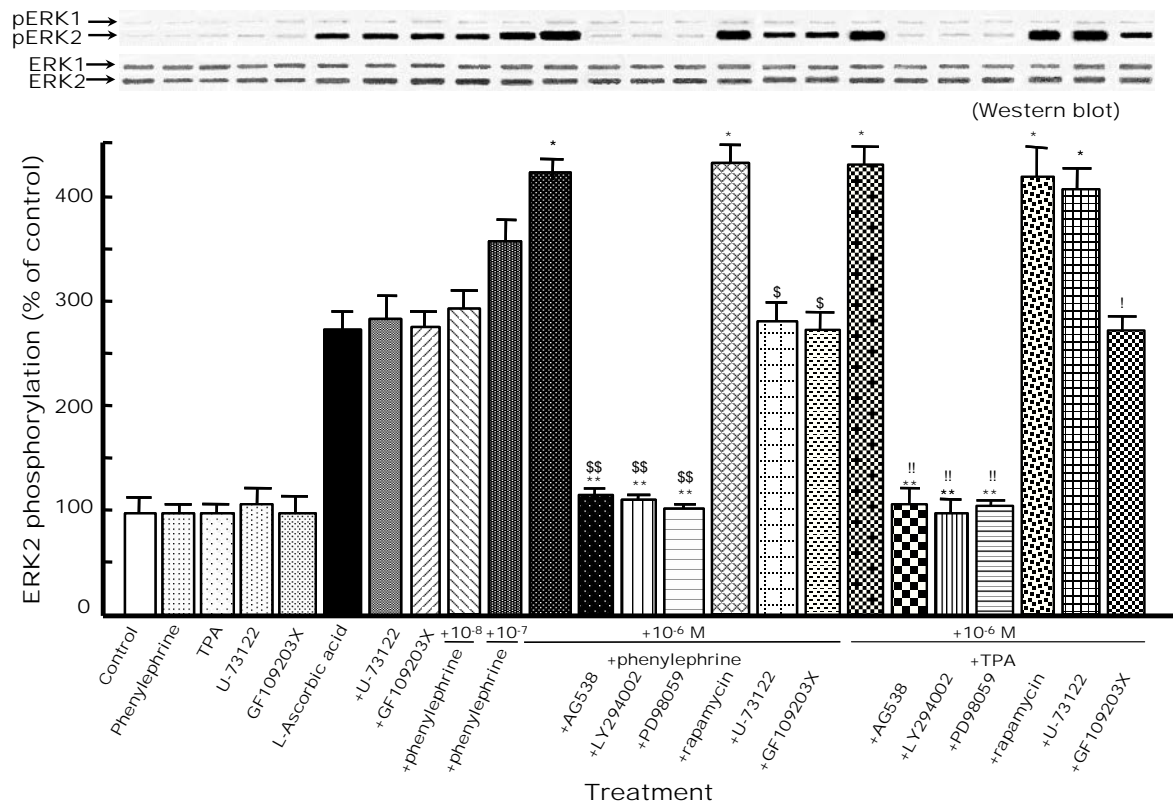
**Fig.5**

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Western blot analysis of pERK1, pERK2, ERK1, and ERK2 in C2C12 cells. The blot shows four rows of bands. The first two rows are labeled pERK1 and pERK2, and the last two rows are labeled ERK1 and ERK2. The lanes are grouped by time points: 0, 1, 3, 5, 10, 20, 30, and 60 minutes. Each time point has four lanes: C (control), C (control), AA (anabolic androgenic steroid), and A+P (anabolic androgenic steroid + protein). The pERK bands show a strong increase in intensity at 5, 10, and 20 minutes, particularly in the A+P lanes. The ERK bands show consistent intensity across all lanes, serving as a loading control.

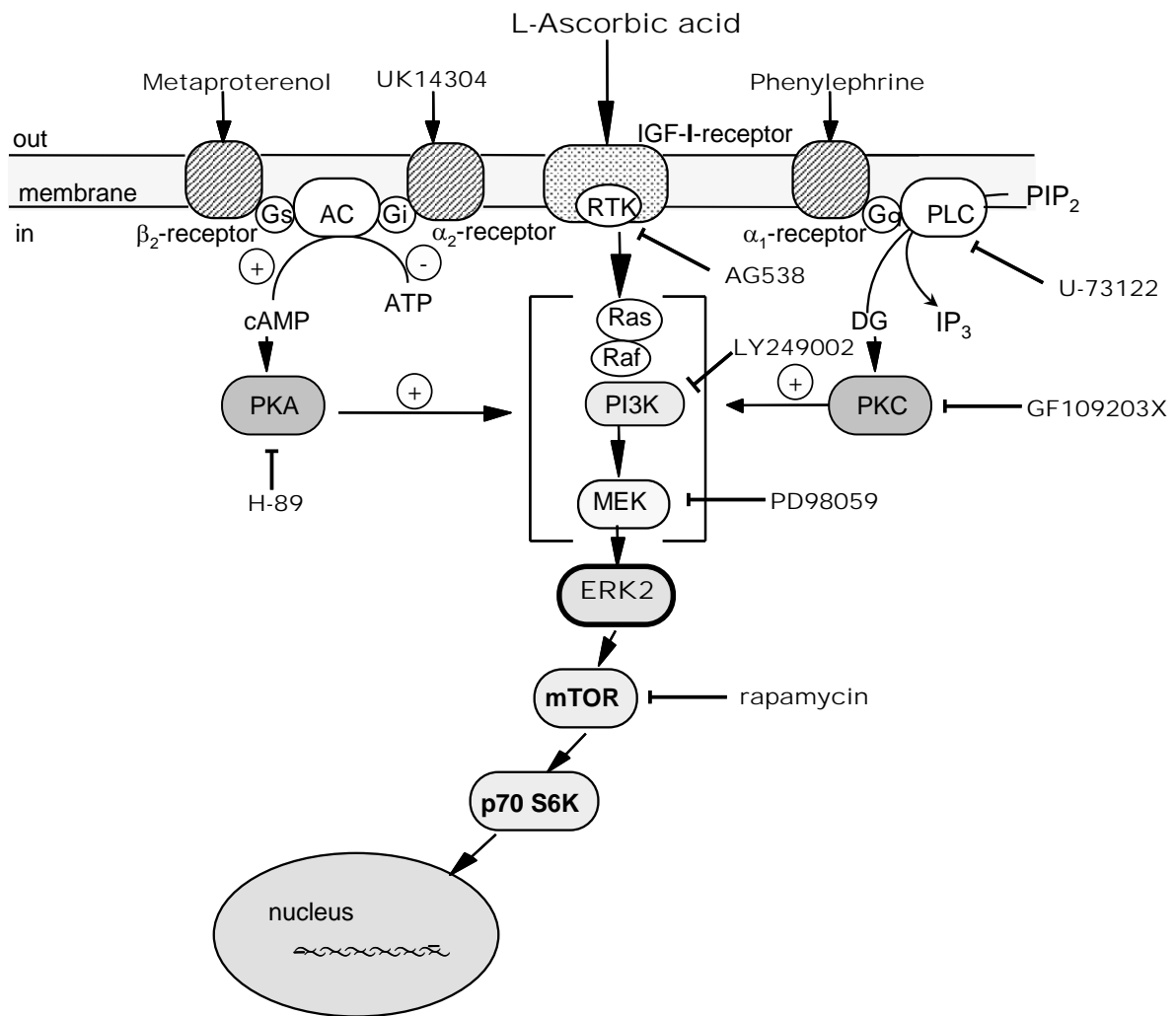


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**Fig.7**

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**Fig.8**

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